Transcriptional Targeting of Adenovirally Delivered Tumor Necrosis Factor α by Temozolomide in Experimental Glioblastoma

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Abstract

Temozolomide is an oral alkylating agent shown to have modest efficacy in the treatment of glioblastoma multiforme. Tumor necrosis factor α (TNF-α) is a polypeptide cytokine with synergistic antitumor activity in combination therapy with alkylating agents. We investigated the combined use of Ad.Egr-TNF, a replication-defective adenoviral vector encoding the cDNA for TNF-α under the control of chemo-inducible elements of the egr1 gene promoter, and intraperitoneal temozolomide in an intracranial human malignant glioma model. In hind limb U87MG xenografts, temozolomide produced a 6.4-fold greater induction of TNF-α after infection with Ad.Egr-TNF compared with Ad.Egr-TNF alone at 96 hours (P < 0.02). TNF-α and temozolomide combination leads to a synergistic decrease in U87 cell viability at 72 hours compared with either treatment alone (P < 0.001). Median survival for animals treated with Ad.Egr-TNF alone, temozolomide alone, and Ad.Egr-TNF/temozolomide was 21, 28, and 74 days, respectively (P < 0.001 by log-rank). Flow cytometric assessment of apoptosis revealed a synergistic increase in U87 cell apoptosis in vitro at 72 hours (P < 0.05), and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) evaluation of tumor sections revealed significantly increased TUNEL-positive cells after combination treatment compared with either treatment alone (P < 0.05). In conclusion, combination treatment with transcriptionally activated intratumoral TNF-α and systemic temozolomide significantly prolongs survival in an experimental glioblastoma multiforme model.

Introduction

Despite aggressive multimodal therapy, median survival of patients with malignant glioma is less than 1 year. Chemotherapy has been shown to modestly increase survival in patients who fail surgery and radiotherapy (1) and temozolomide, an oral alkylating agent, has recently been favored in both the recurrent and upfront setting (2, 3). This improvement in patient survival with chemotherapy, however, remains on the order of weeks due to both an inherent chemotherapeutic drug resistance of glioma cells and an inability to deliver high concentrations of chemotherapeutic drug because of systemic and neurologic toxicity. Chemo/radiotherapy-activated gene therapy is a developing treatment paradigm that attempts to improve the therapeutic index of a variety of potentially therapeutic compounds. Tumor necrosis factor α (TNF-α) is a cytokine with significant antiangiogenic activity (4). Although toxicity has limited its systemic use (5), a synergistic antitumor effect is seen when it is used at high concentrations in regional perfusion strategies with alkylating agents (6). The replication-defective adenoviral vector Ad.Egr-TNF consists of a chimeric gene encoding CARG elements of the chemoinducible/radioinducible early growth response-1 (egr1) gene promoter ligated upstream to the human TNF-α cDNA. This vector has been shown to be induced to express TNF-α by the drug cisplatin (7). In this study, we show that temozolomide can induce the intratumoral (i.t.) expression of TNF-α from Ad.Egr-TNF and that combination treatment with this vector and temozolomide leads to a significant prolongation in survival in an intracranial xenograft model of malignant glioma. The primary mechanism of this antitumor effect appears likely to be due to a synergistic enhancement of tumor cell apoptosis by the combination of TNF-α and temozolomide.

Materials and Methods

Animals, Cells, and Vector

Six- to 7-week-old female athymic nude mice (Frederick Cancer Research Institute, Frederick, MD) were used, and surgery was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Chicago. The human glioblastoma cell line, U87 MG, was cultured in DMEM (Invitrogen Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C and 5% CO2. Ad.Egr-TNF (ViraQuest Inc., North Liberty, IA) was stored at −80°C and diluted in formulation buffer to the appropriate concentration. Temozolomide (Schering Corp., Kenilworth, NJ) was dissolved in DMSO with the final concentration not exceeding 0.1% (v/v).

Tumor Necrosis Factor α Induction In vitro

U87 cells (106) were plated and incubated overnight. The cells were then infected with Ad.Egr-TNF at 100 multiplicities of infection for 3 hours at 37°C. After incubation, 3.8 mL of complete medium with or without temozolomide was added. Conditioned media were harvested at 48 hours after treatment, and human TNF-α production was quantified using a Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D System, Inc., Minneapolis, MN).

Tumor Necrosis Factor α Induction In vivo

U87 cells (5 × 105) in 100 μL of DMEM were injected subcutaneously (s.c.) into the right hind limb of nude mice. When tumors reached an average size of 200 mm3 (length × width × thickness/2), the tumor-bearing mice were randomized into four groups: (1) untreated control, (2) Ad.Egr-TNF alone, (3) temozolomide alone, and (4) Ad.Egr-TNF and temozolomide. Ad.Egr-TNF was injected i.t. at a dose of 2 × 1010 particle units (pu) each day. Two doses of temozolomide were given: 2.5 and 5 mg/kg/d by intraperitoneal (i.p.) injection 3 hours after vector. Four consecutive daily i.t. and i.p. injections were given, and control animals received i.t. and i.p. serum-free medium. Animals were euthanized on days 2 and 4 (i.e., 48 and 96 hours after treatment initiation), and tumors were harvested, snap-frozen in liquid nitrogen, and homogenized in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 10 mmol/L Tris (pH 7.5), 5 mmol/L EDTA (pH 7.5), 100 mmol/L phenylmethi...
RNase (1 mg/mL) for 30 minutes at 37°C and incubated at 37°C.

Guide technique (11). On day 5, mice were randomized into four groups as cells were inoculated into the right caudate nucleus on day 0 using a screw

H9262 temozolomide (100 μmol/L) was calculated and plotted. For intracranial experiments, 5 × 10⁶ U87 cells (10⁵) were plated overnight at 37°C and fixed in ice-cold 70% (v/v) ethanol. The cells were washed twice, incubated in RNase (1 mg/mL) for 30 minutes at 37°C, and then incubated in propidium iodide solution (100 μg/mL) for 30 minutes at 4°C. Flow cytometric analysis was performed on a FACSort instrument (Becton Dickinson Immunocytometry Systems, San Jose, CA), and the data were analyzed using the CellQuest software (Becton Dickinson).

Annexin V Binding. At 72 hours, cells were washed in PBS and incubated in the dark for 15 minutes with binding buffer containing 5 μL of Annexin V-FITC and 5 μL of propidium iodide (Annexin V-FITC apoptosis detection kit II; ref. 12). The data were analyzed by Flowjo analysis software (Tree Star Inc., Ashland, OR).

Histologic Analysis

(1) Paraffin embedded brains were sectioned (8 μm), stained with hematoxylin and eosin, and analyzed in a blinded fashion.

(2) TUNEL assay was performed in accordance with the manufacturer’s instructions (Chemicon, Temecula, CA) and analyzed blindly at ×400 mag-

nification by use of a computer-aided light microscope with reconstruction software (Neurolucida, Microbrightfield, VT). Number of TUNEL-positive cells per 10⁻⁴ mm² was documented.

Statistical Analysis

Results are expressed as mean value ± SD. Statistical significance was taken as P < 0.05 using a one-tailed Student’s t test. Analysis of variance (ANOVA) was also used. Kaplan-Meier survival curves were plotted for the intracranial experiment and analyzed by the log-rank method.

Results

Tumor Necrosis Factor α Induction and Assessment of U87MG Cell Viability. We initially investigated whether temozolomide could induce the expression of TNF-α from U87 cells infected with Ad.Egr-TNF. In vitro, there was no TNF-α detected in the untreated control or temozolomide alone cells, however, after Ad.Egr-TNF infection, 100 μmol/L temozolomide induced a 2.3-fold increase in TNF-α expression compared with cells infected with vector alone (Fig. 1A). We confirmed induction in vivo using hind limb xenografts; again there was no TNF-α detected in the untreated control or temozolomide alone animals, however, after combination treatment with Ad.Egr-TNF/temozolomide, 287 ± 111 pg TNF-α/mg protein were detected at 96 hours—6.4-fold the value in the Ad.Egr-TNF alone animals (n = 3 animals per group, P = 0.02; Fig. 1B).

To determine
whether this combination treatment strategy could result in a significant antitumor response, the cytotoxic effect of TNF-α and temozolomide on glioma cell viability was evaluated in vitro. TNF-α (10 ng/mL) and 100 μmol/L temozolomide alone had minimal effects on U87 cell viability; however, combination treatment lead to a significant reduction in cell viability, the magnitude of which was greater than the sum of the reductions of either treatment alone. Support for a synergistic interaction between TNF-α and temozolomide was observed after ANOVA assessment (P = 0.0016; Fig. 2A and B).

**Investigation of the Antitumor Efficacy of Ad.Egr-Tumor Necrosis Factor and Temozolomide.** Having demonstrated induction of TNF-α and glioma cytotoxicity in vitro, we next evaluated the antitumor efficacy in hind limb s.c. xenografts. There was no significant effect on growth kinetics in tumors treated with vector alone compared with the untreated control animals; however, fractional tumor volume was significantly smaller in the combination treatment group compared with the temozolomide alone group (P < 0.02 at day 20; Fig. 3A). We next studied whether this treatment strategy was effective in an intracranial xenograft model. Nude mouse survival was recorded after treatment, and although 20 mg/kg temozolomide alone prolonged median survival over untreated control and Ad.Egr-TNF alone animals (28 days versus 18 and 21 days, respectively), there were no mice alive past day 48. However, after combination treatment with Ad.Egr-TNF/temozolomide, median survival was significantly increased to 76 days (P < 0.001 by log-rank compared with temozolomide alone; Fig. 3B). The animals in all of the treatment groups appeared healthy. Histologic assessment of intracranial sections showed decreased cell density in the combined treatment group with minimal oligodendroglial toxicity, and most significantly, there was no increase in tumor necrosis when compared with either treatment alone (data not shown).

**Evaluation of Apoptosis.** Because histologic analysis showed that necrosis was not the antitumor mechanism in vivo, we were interested in whether enhancement of apoptosis was a contributing factor. We initially used flow cytometric analysis of U87 cells to assess the fractional DNA content after treatment. As expected, TNF-α alone had minimal effect on U87 cell apoptosis (13), and temozolomide alone lead to an increase in the percentage of cells in G2-M phase (9), however, combination treatment with TNF-α and temozolomide lead to a significant increase in the sub-G1 (hypodiploid/apoptotic) peak at 72 hours compared with either treatment alone (P < 0.05; Fig. 4A). We confirmed apoptosis with annexin V staining of U87 cells; combination treatment led to a 9- and 3.3-fold increase in annexin V-positive cells compared with those treated with TNF-α and temozolomide alone, respectively, at 72 hours (Fig. 4B). The interaction between the two treatments leading to apoptosis was determined to be synergistic, as assessed by ANOVA (P < 0.05). Having demonstrated that the TNF-α and temozolomide combination results in apoptosis in vivo, we were interested in whether this phenomenon was also evident in vivo. Using TUNEL evaluation of intracranial tumor sections, we specifically looked at apoptosis during the early stage of treatment (day 7). Tumors treated with Ad.Egr-TNF/temozolomide combination had significantly more TUNEL-positive cells than those treated with either temozolomide or Ad.Egr-TNF alone (110 ± 77 versus 14 ± 12 and 13 ± 13 TUNEL+ cells/10 4 mm², respectively, P < 0.05; Fig. 4C).
The magnitude of the increase in apoptosis after both TNF-α and temozolomide staining with propidium iodide. The increase in vascular permeability leading to an increase in i.t. drug delivery to a target tumor has been shown to be due to an increase in tumor necrosis possibly due to an increase in tumor necrosis factor α (TNF-α). The therapeutic benefit reported in our experiments. A therapeutic increase in tumor cell apoptosis has been speculated to be a desirable goal of novel glioma therapies (18), particularly because tumor necrosis has been associated with a significantly worse prognosis in glioblastoma multiforme patients (19). However, additional studies are necessary to determine the mechanism involved in the induction of apoptosis and whether treatment-induced apoptosis yields a greater therapeutic ratio in malignant glioma than therapeutically induced necrosis.

Mortality from malignant glioma is related primarily to recurrent disease, which is almost universally local (nonmetastatic) in nature (20). For this reason, these tumors are an ideal target for such a regionally activated treatment strategy. The considerable antitumor efficacy demonstrated in this series of experiments provides preliminary support for the potential use of this treatment paradigm in recurrent glioma patients. Although the animals in the treatment groups were noted to be healthy, formal toxicity studies will need to be performed before making conclusions regarding the potential toxic effects of this treatment.

References

8. Raza SM, Lang FF, Aggarwal BB, Fuller GN, Wildrick DM, Sawaya R. Necrosis and apoptosis after high-dose TNF-α and temozolomide treatment together is greater than the sum of the increases after TNF-α alone nor temozolomide alone results in apoptosis in glioma cells (9, 17). Considered together, these data strongly suggest that there is a direct interaction between TNF-α and temozolomide in glioma cells that enhances apoptosis, resulting in the therapeutic benefit reported in our experiments.

Discussion

Our data demonstrate a novel use of transcriptionally targeted gene therapy to enhance the effect of a commonly used antiangiogenic agent, temozolomide. Although one advantage of this drug is its relatively mild side effects (14), the maximal dose that can be safely administered is still limited by hematologic toxicity (15). Herein, we report that the therapeutic index of temozolomide can be greatly enhanced when it is used in a combination treatment strategy with a drug that enhances the therapeutic ratio in malignant glioma.

In isolated limb perfusion studies, where high dose TNF-α is used with an alkylating agent, a synergistic interaction has been documented that has been shown to be due to an increase in tumor necrosis possibly due to an increase in tumorigenic activity leading to an increase in i.t. drug concentration (16). A similar pattern of tumor necrosis has also been observed when radiotherapy is combined with Ad.Egr-TNF in a flank glioma model (8). Although tumor necrosis was not observed histologically in our experiments, we did observe a significant increase in tumor cell apoptosis both in vitro and in vivo. However, we and others have shown that neither TNF-α alone nor temozolomide alone results in significant apoptosis in glioma cells (9, 17). Considered together, these data strongly suggest that there is a direct interaction between TNF-α and temozolomide treatment is needed to determine the mechanism involved in the induced apoptosis and whether treatment-induced apoptosis yields a greater therapeutic ratio in malignant glioma than therapeutically induced necrosis.

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